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The emergence of enterovirus D68 in a Dutch University Medical Center and the necessity for routinely screening for respiratory viruses

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ABSTRACT

Background: Since August 2014, an increase in infections caused by enterovirus D68 (EV-D68) was reported in the USA and Canada, for the most part in children presenting with severe respiratory symptoms.

Objectives: To determine whether an increase in severe EV-D68 respiratory infections was observed in our region.

Study design: Samples from patients with respiratory symptoms were screened for viral pathogens, including rhinovirus and enterovirus. Subsequently, samples positive for rhinovirus and enterovirus were routinely sequenced for phylogenetic analysis. Furthermore, an additional method was used to detect EV-D68 specifically.

Results: During the first three quarters of the year 2014, 1896 respiratory samples were analyzed; 39 (2%) of them tested positive for enterovirus. Eighteen samples tested positive for EV-D68, obtained from 16 different patients admitted to our hospital. Eleven were children below the age of 18, of whom five children needed intensive care treatment. The remaining five samples were from adults, who all had an underlying disease; three were transplant patients (heart, lung and renal transplantation), the other two had an underlying lung condition (COPD, asthma). Phylogenetic analysis showed a close relationship with the strains circulating currently in the USA, all belonging to the known EV-D68 genetic subtypes.

Conclusions: We observed an increase of EV-D68 infections in our population, both in children as well as in adult. In 2014 there have been 16 cases so far, compared to none in 2011 and 2013 and a single case in 2012. Phylogenetic analysis identified two similar clusters as shown in the USA and Canada.

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1. Background

Since August 2014, an increase in infections caused by enterovirus D68 (EV-D68) has been reported in the USA and Canada. In this outbreak, the majority of samples analyzed are from children with severe respiratory illness, many of whom have symptoms of wheezing. Also, some fatalities have been documented [1].

In the years 2009 and 2010, our hospital in the Northern part of the Netherlands was confronted with an increase in respiratory illness, which was not only seen in our tertiary care hospital, but also regionally [2]. An outbreak of EV-D68 was shown to

be the cause of the increased incidence of respiratory infections. Because EV-D68 was not detected by our routine respiratory panel at that time, we added an enterovirus PCR into our routine screening panel of 14 respiratory viruses (Influenza A, Influenza B, parainfluenzavirus 1–4, coronaviruses OC43, NL63 and 229E, adenovirus, bocavirus, RSV-A, RSV-B and human metapneumovirus). Moreover, we expanded our testing by routinely sequencing all enterovirus and rhinovirus strains to identify virus strains and to chart outbreaks and transmission patterns. Adding to this sequencing strategy, we included noroviruses as well as parechoviruses. Nationally, a structure called TYPENED was available through the Dutch National Institute of Health (RIVM) [3]. Furthermore, by using our regional sequencing and epidemiological strategy, called REGIOTYPE, we were able to speed up the flow of information from our region, including hospitals and Public Health Services covering the (not densely populated) Northern part of the Netherlands. The aim of this REGIOTYPE strategy is to provide a rapid sequencing

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service for the whole region. This not only completes our diagnostics with sequencing data, but as clinical data are fed back into our database, we create a source of information which serves to obtain more insights in epidemiology patterns.

Recently, the European Center for Disease prevention and Control (ECDC) concluded in a “rapid risk assessment” [4] that no epidemic clusters of severe disease caused by EV-D68 in EU/EEA countries were observed and that a moderate risk of EV-D68 was expected. The ECDC rapid risk assessment attributes the low level of reporting of EV-D68 to the low number of laboratories which routinely screen respiratory samples for EV-D68. Subsequently, only a small number of EV-D68 cases (11) are observed in the Netherlands during the enterovirus ‘peak season’ in 2014, through both the entero-surveillance and the ILI/ARI surveillance networks [5]. However, we are observing an increase in infections caused by EV-D68 in our tertiary care hospital since May 2014, similar to the outbreak in the USA and Canada. To improve the specific diagnostics for EV-68, we developed a specific real-time PCR method.

2. Objectives

To determine the circulation of EV-D68 in our region, during a time when an outbreak of this virus is ongoing in North America, and to develop an EV-D68 specific PCR method.

3. Study design

3.1. Patients and sample collection

Samples from patients with respiratory symptoms, are routinely screened for several viruses, including influenza A and B, RSV type A and B, metapneumovirus, parainfluenzavirus type 1–4, coronavirus (OC43, 229E and NL63), adenovirus, bocavirus, rhinovirus and enterovirus. During the first three quarters of the year 2014, 1896 respiratory samples were analyzed. Samples that tested positive for enterovirus were selected for sequencing.

3.2. Detection of enterovirus/rhinovirus

All primary patient materials sent to our laboratory for analysis of respiratory viruses, are routinely screened for rhinovirus and enterovirus by a laboratory developed real-time PCR that amplifies a 116bp fragment of the 5' NTR region of both rhinovirus and enterovirus. Differentiation between both viruses is done by the use of highly specific probes containing locked nucleic acids (LNA) at the 5' terminus (Table 1). RNA was extracted from 190 µl sample with the addition of 10 µl Internal Control, phocine distemper virus (PDV), using the NucliSense EasyMag (bioMérieux, Lyon, France). PCR was performed in a total reaction volume of 25 µl using 10 µl RNA, 1xTaqMan® Fast Virus 1-Step Master

Mix (Applied Biosystems, Foster City, CA, USA), 750 nM forward and reverse primer for Rhinovirus/Enterovirus, 250 nM Rhinovirus probe, 125 nM Enterovirus probe each, 300 nM forward and reverse primer for PDV, 100 nM probe for PDV and DNase/RNase free water (Sigma). Reactions were run on an ABI7500 using the profile of 2 min 50 °C, 20 s 95 °C, followed by 45 cycles of 3 s 95 °C and 32 s 60 °C.

3.3. Sequencing strategies and phylogenetic analysis

Rhinovirus and enterovirus positive samples were routinely typed by sequencing. Identification of the rhinovirus strains was done by amplification and sequencing of a 549 nucleotide fragment spanning the hyper variable part of the 5' NTR, the entire VP4 gene and the 5' terminus of the VP2 gene with primers described by Savolainen et al. [6]. Identification of the enterovirus strains was done by amplification and sequencing of 350–400 basepairs part of the VP1 gene as has been described by Nix et al. [7].

An automated DNA sequencer was used (ABI 3130XL). Sequence data were analyzed with Sequencing Analysis (version 5.3; ABI) and subsequently phylogenetic analysis was performed using BioNumerics Software 6.6 (Applied Maths, Sint-Martens-Latem, Belgium).

3.4. -Development of an EV-D68 specific real-time PCR

To support initiatives for the rapid and specific detection of EV-D68, a Real-Time PCR was developed for fast detection of EV-D68. Primers and probes were developed using a ClustalW2.0 alignment of all currently available 5' Non Coding Region (NCR) sequences in the NCBI database and Primer Express 3.0 software (Applied Biosystems).

Twenty-two samples, spanning a period from 2010 to 2014, previously detected by RT-PCR and identified with EV-D68 by sequencing, were tested for validation. For determining specificity, six currently circulating human rhinovirus (HRV) strains of the genogroups A, B and C (HRV-A38, HRV-A43, HRV-B17, HRV-B70, HRV-C2 and HRV-C18) and 11 samples from 2014 containing different enterovirus species (CV-A2, CV-A4, CV-A6, CV-A10, CV-A11, CV-B4, E-3, E-16, E-25, EV-C105 and EV-C117) were included in testing, as well as five reference strains provided by the Dutch National Institute of Health (CV-A12, CV-A15, E-5, E-11 and the EV-D68 reference Fermon strain).

Sample types included were sputum, nasopharynx, flocked swab, stool and cerebrospinal fluid.

RNA was extracted as described previously. PCR was performed in a total reaction volume of 25 µl using 10 µl RNA, 1xTaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems, Foster City, CA, USA), 600 nM forward and reverse primer for EVD68, 200 nM EV-D68 probe each (Table 3) and DNase/RNase free water (Sigma).

Table 1
Primers and probes for RT-PCR and sequencing of enterovirus D68.

PCR	Oligo	Sequence (5' → 3')	Target	Orientation	Reference
Rhinovirus/enterovirus RT-PCR	HRV/HEVfw	CGGCCCTGAATGYGG	5' NTR rhinovirus/enterovirus	Sense primer	LDT
	Rhi-asense	TGGAAACACGGACACCCAA	5' NTR rhinovirus/enterovirus	Anti-sense primer	LDT
	HEV1-lna-pr4	Cy5-YGTGGCGGAACCGA-BHQ3	5' NTR enterovirus	Probe	LDT
	HEV2-lna-pr2	Cy5-YGCAGCGGAACCGACT-BHQ3	5' NTR enterovirus	Probe	LDT
	Rhino-lna-pr2	FAM-YGGGAYGGGACCACT-BHQ1	5' NTR rhinovirus	Probe	LDT
IC RT-PCR	PDV fwd	CGGGTGCCTTTTACAAGAAC	Haemagglutinine PDV	Sense primer	Clancy et al. [10]
	PDV rev	TTCTTCTCAACCTCGTCC	Haemagglutinine PDV	Anti-sense primer	Clancy et al. [10]
	PDV_MGB_NED	NED-AAGGGCCAATTC-MGB	Haemagglutinine PDV	Probe	Clancy et al. [10]
EV-D68 specific RT-PCR	EV-D68 FW	TGTTCACCGGTGAAACAA	5' NTR EVD68	Sense primer	LDT
	EV-D68 RV	TGTCTAGCGTCTCATGGTTTTCAC	5' NTR EVD68	Anti-sense primer	LDT
	EV-D68 Probe	VIC-TCCGCTATAGTACTTCG-MGB	5' NTR EVD68	Probe	LDT
	EV-D68 Probe	VIC-ACCGCTATAGTACTTCG-MGB	5' NTR EVD68	Probe	LDT

Table 2

Patient characteristics of patients admitted with EV-D68 in chronological order.

Patient	Sex	Age	Date	Symptoms	ICU/days	Underlying condition
1	F	1y/11m	February 2014	Moderate bronchiolitis-type respiratory illness	No	None
2	F	1y/7m	May 2014	Severe respiratory illness, intubation, mechanical ventilation	Yes/4	None
3	F	6m	June 2014	Mild cold symptoms	No	Congenital heart disease
4	M	3y/9m	June 2014	Severe respiratory illness, intubation, mechanical ventilation	Yes/8	Sickle-cell anemia
5	M	65y	July 2014	Moderate wheezing and cough	No	Lung transplantation
6	M	1y	July 2014	Severe respiratory illness, intubation, mechanical ventilation	Yes/5	None
7	M	3y/10m	July 2014	Severe respiratory illness, intubation, mechanical ventilation	Yes/1	Ex-premature
8	M	6m	August 2014	Mild cold symptoms	^a	Congenital heart disease
9	M	14y	August 2014	Mild cold symptoms	^a	Traumatic brain injury
10	F	1y/6m	August 2014	Mild cold symptoms	No	Epilepsy
11	M	1m	August 2014	Severe respiratory illness, intubation, mechanical ventilation	Yes/1	Ex-premature
12	F	22y	August 2014	Cough, SOB, fatigue	No	Heart transplantation
13	F	44y	August 2014	Cough, SOB	No	COPD
14	M	1y/5m	September 2014	Moderate wheezing, cough	No	Tracheomalacia, congenital heart disease
15	F	63y	September 2014	Asthma exacerbation	No	Asthma
16	F	53y	September 2014	Wheezing, pneumonia	No	Kidney transplantation

SOB, shortness of breath.

^a These patients were admitted to ICU for reasons other than respiratory illness.

Reactions were run on a ABI7500 as follows: 15 min 50 °C, 20 s 95 °C, followed by 45 cycles of 5 s 95 °C, 5 s 50 °C and 45 s 60 °C.

to GenBank, accession numbers: KM887894–KM887906 and KM924544–KM924547.

4. Results

Since the beginning of 2014, 1896 respiratory samples were tested, of which 39 (2%) were positive for enterovirus. Eighteen samples tested positive for EV-D68, obtained from 16 different patients admitted to our hospital. (Table 2). Eleven cases were children of whom five presented with severe respiratory illness, requiring intubation and mechanical ventilation. Of these five severely ill children, two (aged 1 year and 1 year/7 months) had been previously healthy and two were ex-prematures (aged 3 years/10 months and 1 month). One of the previously healthy children (patient 6) was given resuscitation prior to admission to the pediatric intensive care unit. One child had sickle cell anemia as underlying condition (3 years/9 months). Two children were admitted with a moderately severe bronchiolitis-type respiratory infection with wheezing and shortness of breath. Four children had mild respiratory infections with common cold-symptoms only. These children all had underlying conditions for which they were admitted to our hospital. Interestingly, two of these children (patient 8 and 9) acquired the infection several days after admission. The sequences of these isolates were identical with the sequence of patient 7 (GenBank Accession numbers: KM887895, KM887898 and KM887899). Both patient 8 and 9, were admitted to the same pediatric intensive care unit during the same period as the admission of patient 7, which suggests nosocomial transmission.

Five cases of EV-D68 infections were from adults. All adult patients had underlying conditions predisposing them to respiratory tract infections. One patient was a heart transplantation patient, one was a lung transplant patient, one was a kidney transplantation patient, one had asthma and one had chronic obstructive pulmonary disease (COPD). All five adults had to be admitted to the hospital with symptoms of cough, shortness of breath and wheezing. The patient with a kidney transplant (patient 16) had radiological signs compatible with pneumonia.

In all 16 cases, the diagnosis of EV-D68 infection was made following a positive PCR result for enterovirus and subsequent sequencing. EV-D68 partial VP1 sequences were submitted

4.1. Epidemiological information since 2009

We tested 1896 respiratory samples in our laboratory during the first three quarters in 2014. 39 (2%) samples were tested positive for enterovirus. Different enterovirus types were isolated, of which 18 samples were EV-D68 (Table 3). The upsurge of EV-D68 corresponds with the regular enterovirus season period in 2014. A similar upsurge occurred in 2009 and 2010, with a peak in September and October. However, in 2011 and 2013, there were no EV-D68 observations at all, while in 2012 only one respiratory sample contained EV-D68 (Fig. 1).

4.2. EV-D68 specific real-time PCR

The EV-D68 test was highly specific, all strains were tested negative for types other than EV-D68 as described in Section 2. The assay was designed in the 5' NCR. All clinical samples of 2014 which tested positive in the standard EV assay and subsequently typed as EV-D68, were also tested positive in the EV-D68 specific PCR.

Table 3

Enteroviruses detected from respiratory samples, UMCG 2014.

Genotype	Number of samples
CV-A4	1
CV-A6	5
CV-A16	1
CV-B1	1
CV-B4	1
E-16	4
E-18	1
E-25	2
EV-C104	1
EV-C105	1
EV-D68	18
ND ^a	3
Total	39

^a ND, not defined.

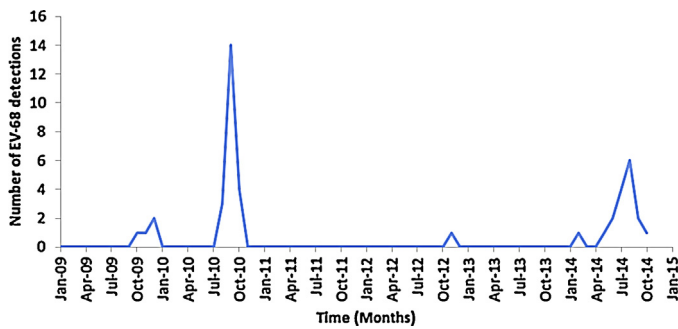


Fig. 1. EV-D68 occurrence in the UMCG 2009–2014.

4.3. Sequence data and information

Phylogenetic analysis shows that 3 out of the 17 sequences correspond with clade A and 14 out of 17 sequences with clade B, as described by Tokarz et al. [8]. None of our sequences corresponded with clade C. USA strains correspond with both, clade A and clade B,

suggesting that similar strains are currently circulating in the USA and Europe (Fig. 2).

5. Discussion

In this paper, we presented 16 cases of respiratory EV-D68 infections in the Northern part of the Netherlands in 2014. These patients were in most cases admitted to the hospital because of a serious respiratory illness. Of these 16 cases, five were children with a life-threatening respiratory illness. We also noted five EV-D68 infections in adults, all with underlying conditions, predisposing to more severe respiratory illness. Three were transplantation patients and two had pulmonary conditions. All of these five adults required hospital care for respiratory infections.

This upsurge of respiratory infections caused by EV-D68 was similar to what was seen in our hospital in 2009 and 2010 [2]. More importantly, the 2014 EV-D68 upsurge in our hospital occurred during a time period while a large EV-D68 outbreak took place in the USA and Canada, involving around a thousand children. Also in the USA, only the children admitted to the hospital were tested and reported. We expect that the number in North America could be

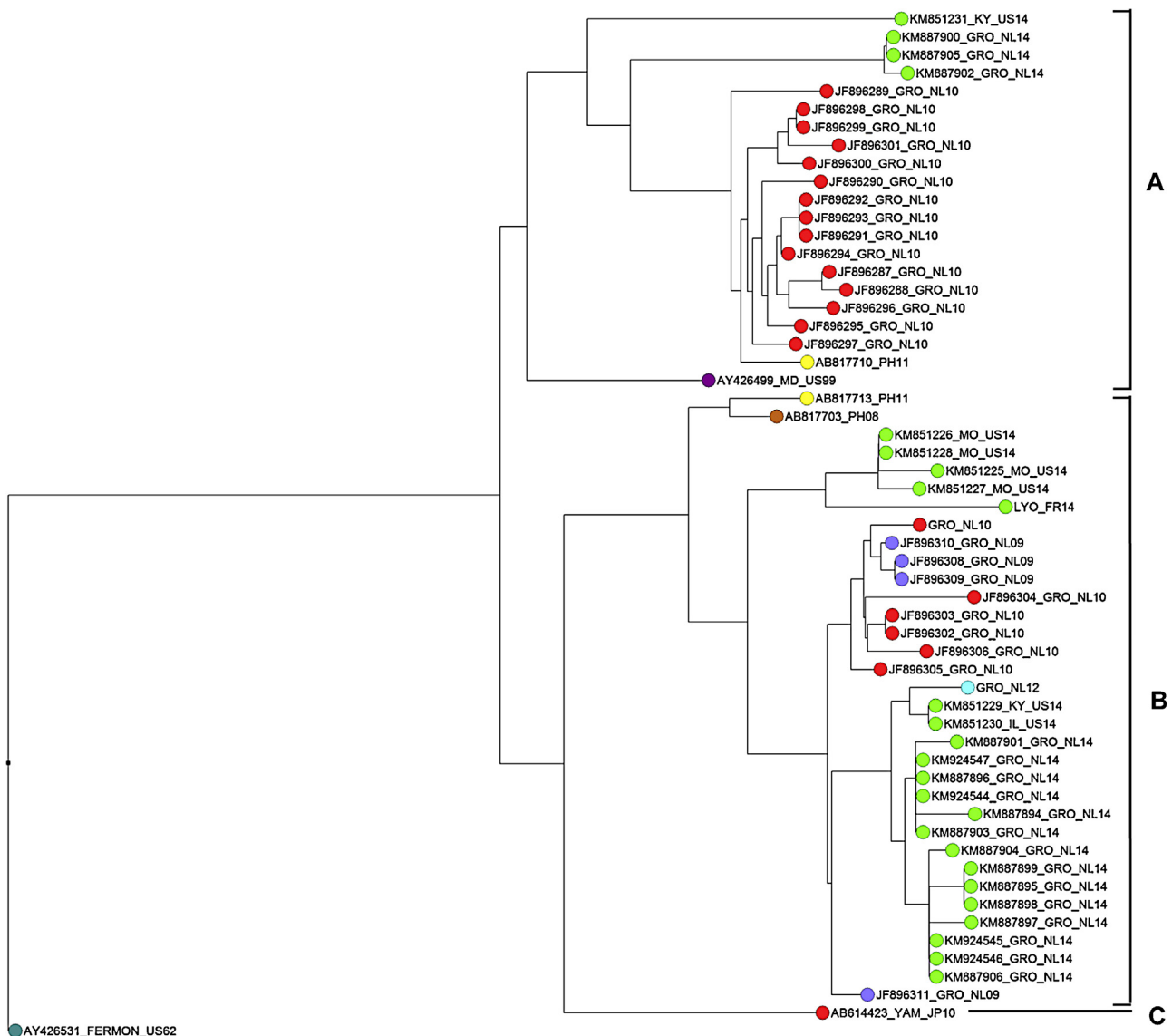


Fig. 2. Phylogenetic tree EV-D68 2009–2014. Node colors – purple: 2009, red: 2010, yellow: 2011, light blue: 2012, green: 2014. Clades A, B and C as defined by Tokarz et al. [8].

higher if the adult population with severe respiratory illness were included.

The incidence of EV-D68 observed in our hospital, which is located in the least densely populated part of the Netherlands, is contrasted by the low number of reported EV-D68 infections in rest of the Netherlands, while no data are known about the current situation in Europe [4,5]. After all, it seems unlikely that EV-D68 is causing problems only in the USA, Canada and the Northern part of Netherlands, without affecting other European regions. In the 2009–2010 upsurge, a number of children became severely ill. Moreover, it was then shown that 21% of the cases were hospital acquired, which highlights the need for testing of not only patients with severe respiratory disease, but also patients with mild symptoms, who may spread the virus. Similarly, in the current 2014 study, we possibly witnessed in-hospital transmission of EV-D68 from one severely ill child to two children who developed mild respiratory symptoms.

The low number of reported EV-D68 infections in Europe may be caused by the overall low circulation of EV-D68. However, it could also be caused by the under-diagnosis of EV-D68 infections due to insufficient sampling of patients with respiratory illness, or an insufficient detection of this virus in routine screening panels, whether using Laboratory Developed Tests (LDTs) or commercial assays. From QCMD quality assurance data, it is known that the respiratory virus testing performed in a number of laboratories failed to detect EV-D68 (Dr. Paul Wallace, personal communication, QCMD, Glasgow, United Kingdom).

The recent ECDC report claims that the circulation of EV-D68 in Europe is low, thus has to be viewed in the context of a near absent detection of this pathogen [4]. In addition, the potential for under-sampling has to be considered. Although no exact data exists on underdiagnoses as yet, it is our experience that clinicians, at least in the Netherlands, are discouraged from performing diagnostic tests for patients presenting with probable viral illnesses because of financial constraints. And if testing is done, only influenza virus and RSV are preferably tested.

A recent initiative by the European Society for Clinical Virology (ESCV) in collaboration with the ECDC was started to investigate the prevalence of EV-D68 in Europe. Further epidemiological information is needed on a larger scale than currently available to determine the circulation for this virus. The implicit risk of focusing on a limited number of viruses for diagnostic purposes, or not performing virological diagnostics at all, is contestable. Patients with an underlying disease, whether children, transplant patients, or patient with a chronic respiratory condition, are not getting optimal care if the diagnostic options are limited or even absent. Therefore, a diagnostic stewardship program should be discussed besides antimicrobial stewardship and an infection control programs, which are currently being introduced in many hospitals [9]. We have documented upsurges of EV-D68 associated with sometimes severe morbidity twice in the last 5 years, i.e. in 2009/2010 and in 2014. It can be anticipated that the virus will reappear regularly and therefore diagnostic assays, whether LDTs or commercial assays, should be able to detect this virus. The EV-D68 specific assay we describe in this paper, may be used for this purpose.

All 16 cases described in this paper were diagnosed as EV-D68 infections by following routine protocols of our laboratory, in

which respiratory samples are tested for enterovirus along with 14 other respiratory targets, and all enterovirus isolates are routinely sequenced. Our sequencing data, compared to the USA sequence data, suggest that similar strains are circulating in the USA and Europe.

In conclusion, by following a routine protocol of screening respiratory samples for enterovirus and sequencing, we not only identified EV-D68 as the cause of 16 respiratory infections in our hospital, but we also were able to show a potential link with the ongoing EV-D68 in North-America. EV-D68 is associated with at times severe respiratory illness. Adequate surveillance for viruses like EV-D68 which can cause severe respiratory illnesses, is therefore desirable for University Medical Centers and Reference Centers. A collaborative approach effort between the diagnostic and reference laboratories could be taken to achieve this. The EV-D68 specific assay we present in this paper may aid in the assessment of the real prevalence of this virus in our population, both in children and adults.

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Competing interests

The authors have no competing interests to report.

Ethical approval

No ethical approval was required for this study.

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